Iron Localization in Pea Plants [1,2]

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Iron chlorosis has often been ascribed to iron inactivation, immobilization, or precipitation within the plant (6, 7, 14, 18, 20). This argument has rested on the assumption that there is no correlation between the chlorophyll content and the iron content of chlorotic plants. Since more recent findings do not support this assumption (1, 11, 12, 13), it is difficult to assess suggestions that chlorosis is the result of iron precipitation in xylem elements (14, 18), nodes (10), high-pH tissue such as phloem or bundle sheaths (20), or translocating tissues in general (7, 9, 19). If the total iron content of a leaf is related to its chlorophyll content, there is no reason to claim localized regions of high iron represent precipitation or inactivation. Before ascribing chlorosis to iron immobilization, more should be known about some fundamental aspects of its transport and distribution in normal, green plants.

A previous study (5) described some of the factors governing iron transport. As a continuation of that study, the localization of absorbed iron was examined by various radioautographic techniques, some of which permitted cellular localization of soluble as well as insoluble iron. These radioautographic results are presented here. While corroborating previous evidence showing the metabolic involvement of root cells in iron transport (5), the radioautographic results shed new light on the cellular distribution of iron in non-chlorotic plants.

Methods

Pea plants (*Pisum sativum*, L., var. Alaska) were used in all of the experiments. The plants were grown in aerated, purified Hoagland solutions as has been detailed elsewhere (5). For an experiment, the plants were transferred to aerated experimental solutions containing Fe^{55} or Fe^{59}. The experimental solutions contained 0.5 ppm Fe, added as $FeCl_3$, and 1 meq/liter $CaSO_4$. Exceptions and specific treatments will be indicated with each experiment below. After experimental treatment, either the entire shoot, pieces of leaves, or root parts were harvested and prepared for radioautography. Roots were always rinsed for 5 seconds in each of three changes of distilled water before harvest.

Macroradioautographs of shoots were prepared by pressing the shoots between a padded weight and a piece of cold (about −75 C) glass supported on dry ice. The shoots froze within seconds, whereupon they were transferred to cold X-ray film and kept frozen until the end of the exposure period. Radioautographs of tissues dried between blotters showed redistributional artifacts similar to those noted by Pallas and Crafts (15).

Pieces of roots or leaves were prepared for microradioautography by quenching them in a freezing mixture cooled to about −175 C with liquid N_2. The mixture contained 8 % (v/v) methylcyclohexane in isopentane and became solid at a temperature 10 C to 15 C lower than isopentane alone. After freezing, the samples were freeze-dried at −45 C using a stream of slightly moistened gas (3). When dry, the samples were embedded in paraffin at 60 C, sectioned and dry-radioautographed (4) so as to localize soluble, as well as insoluble iron.

Results

The first experiment was designed to localize newly absorbed iron and follow its distribution in the root. Nine-day-old plants were placed in Fe^{59} labelled solutions of $FeCl_3$ for 4, 40, 180, and 1,440 minutes. Half the plants used had been grown in solutions to which 0.5 ppm Fe as $FeCl_3$ had been added for 48 hours prior to the experiment. The other half had grown in the absence of iron. The specific activities of the experimental solutions were

Fig. 1. Microradioautographs of roots which had been in Fe^{59} labelled $FeCl_3$ for 4 minutes (1a, d, & f), 180 minutes (1b & e), and 1,440 minutes (1c). Before being placed in the labelled Fe solutions the plants in 1a, b, & c had grown in Hoagland solution containing 0.5 ppm Fe as $FeCl_3$; the plants in 1d, e, & f had grown in the absence of iron. All of these photographs were taken with dark-field illumination so that the silver grains in the radioautographs stand out sharply against the barely visible outlines of the apposed cross-sections. a & d, 100 : 1; b, c, & e 180 : 1; f, 500 : 1.
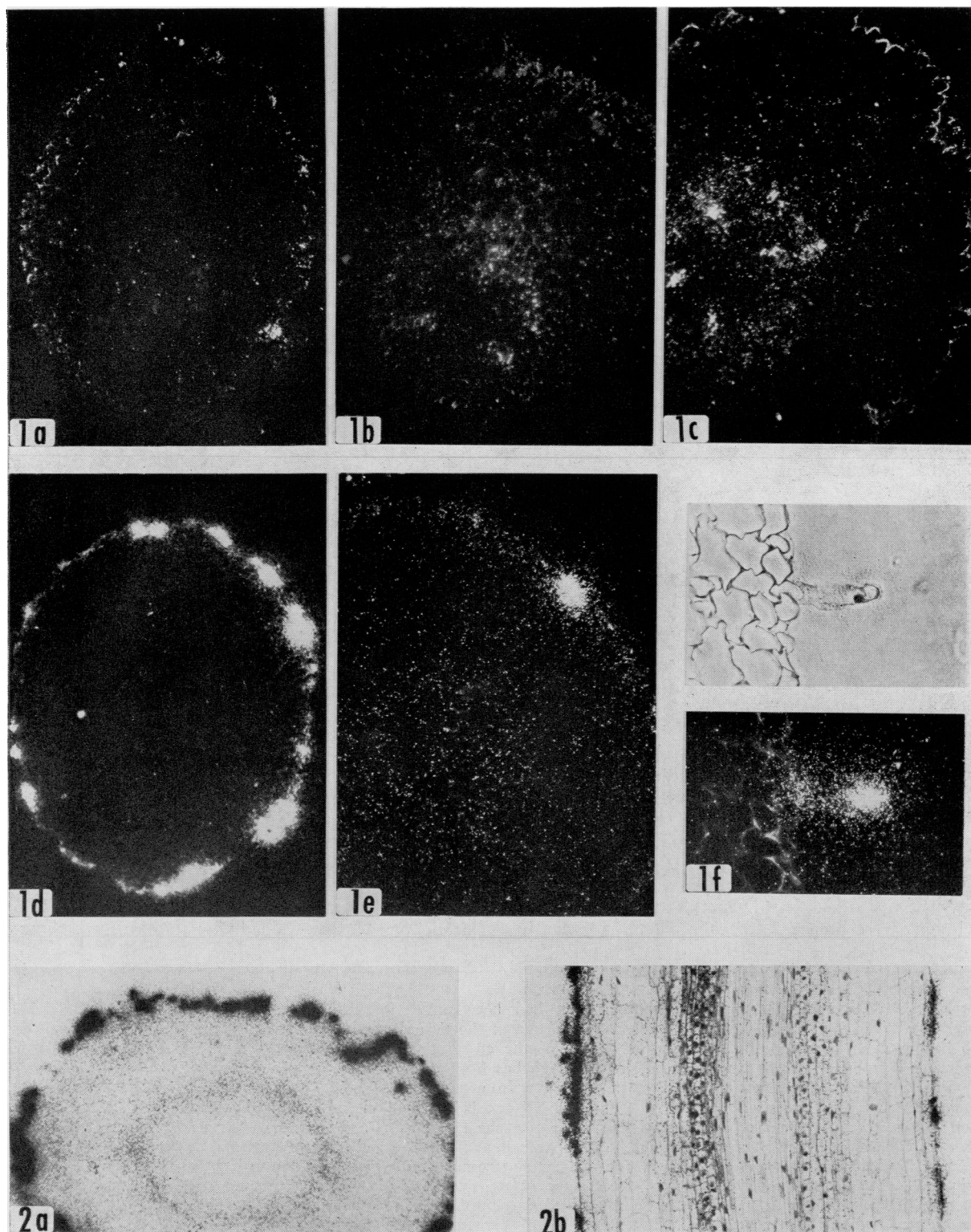
Fig. 2. Microradioautographs of roots which had been in Fe⁵⁹ labelled FeCl₃ for 14 hours. In 2a the root cross-section has been washed off the radioautographic plate during processing so that only the general pattern of Fe⁵⁹ distribution is obvious. The regions containing the greatest amounts of Fe⁵⁹ can be discerned more exactly from the radioautograph of the longitudinal section in 2b, which was stained with Delafield's hematoxylin. 75:1.

adjusted on the basis of preliminary experiments so that all of the plants contained about the same amount of Fe^{59} at harvest. Thus, the solutions to be used for 4, 40, 180, and 1,440 minutes contained, respectively 1.5, 0.7, 0.5, and 0.2 μc $Fe^{59}/\mu g$ Fe. After the designated time, the roots were harvested and prepared for microradioautography. A few serial sections from each of the various treatments were mounted on the same radioautographic plate. Cross sections of the vacuolated portion of secondary roots, about one centimeter from the tip, were used since this is the region of the root in which the xylem is well differentiated and which is presumed to function in the transport of inorganic nutrients (23).

The results are presented in figure 1. The important features to note are: A, Plant roots which had received iron for 48 hours prior to the experiment were sparsely coated with radioactive iron after 4 minutes in Fe^{59} (fig 1a). Little radioactivity could be detected within the root until the plant had remained in the Fe^{59} solution for 40 minutes (not shown). Plants harvested after 180 minutes clearly showed Fe^{59} in the xylem and phloem, and lesser amounts in the cortex (fig 1b). After 1,440 minutes Fe^{59} was distributed throughout the root cross section, though the greatest concentrations were observed in the xylem and in those cell layers contiguous to the endodermis (fig 1c). B, Plant roots which had grown in the absence of iron were completely coated with radioactive iron after 4 minutes in Fe^{59} (fig 1d); particularly heavy activity was frequently associated with root hairs (fig 1f). Again, little activity was detected within the root after the 4-minute treatment, though activity in the peripheral cortical cells was visible after the 40 minute treatment (not shown). After 180 minutes, Fe^{59} was detectable throughout the root section, though not always in the xylem (fig 1e). The cell layers adjacent to the endodermis seemed to have the greatest concentrations of Fe^{59}.

In a second experiment, microradioautographs of tap root tips of 2-day-old plants grown in iron-free solutions were prepared after the plants had been in $Fe^{59}Cl_3$ (0.5 $\mu c/\mu g$ Fe) for 14 hours. The overall distribution of Fe^{59} could be seen most clearly in portions of the radioautograph where the sections themselves had been inadvertently washed off the photographic emulsion (fig 2a). Again, high concentrations of Fe^{59} were noted on the root surface and in cell layers adjacent to the endodermis. Examination of other radioautographs (fig 2b) indicated the region of high Fe^{59} concentration within the root was associated with the more densely cytoplasmic cells on both sides of the endodermis. Plants were also placed in solutions of high specific activity iron (23 $\mu c/\mu g$ Fe; total iron 0.03 ppm) and solutions of iron ethylenediamine tetraacetate (FeEDTA) labelled with Fe^{59} (0.5 $\mu c/\mu g$ Fe). In both cases, the distribution of Fe^{59} in roots was similar to that in the roots (fig 2) treated with the lower specific activity $Fe^{59}Cl_3$.

In a third experiment, 9-day-old plants were placed for 3 hours in a solution of labelled $FeCl_3$ to which 5×10^{-5} M 2,4-dinitrophenol was added. Radioautographs of the roots (not shown) indicated that 2,4-dinitrophenol inhibited the movement of iron into the root but did not prevent its deposition on the root surface.

In a fourth experiment, both macro- and microradioautographs were used to follow the distribution of newly absorbed iron in the leaf. In this experiment, all of the plants were grown in unlabelled iron for 48 hours before being fed labelled iron for either 180 minutes or 1,440 minutes. The experimental methods were identical to those used in the first experiment, except that plants intended for macroradioautography were fed $Fe^{55}EDTA$ (1 $\mu c/\mu g$ Fe) rather than Fe^{59}.

The results of the macroradioautographs are shown in figure 3. The spotty distribution of Fe^{55} seen in this figure was observed in all normal plants, even when, as in other experiments (2), uptake was from $Fe^{55}EDTA$.

After the tissues were used to produce the radioautograph shown in figure 3b, they were cleared in alcohol and NaOH, stained with safranin, and photo-

Fig. 3. Macroradioautographs of plants which had been in Fe^{55} labelled $FeCl_3$ for 180 minutes (3a) and 1,440 minutes (3b & c). In 3c, the enlarged image of a portion of the tissues previously used to make the radioautograph in 3b has been superimposed on the enlarged image of the corresponding portion of the radioautograph. The arrow indicates the portion of the radioautograph in 3b which has been used in 3c.

Fig. 4. Microradioautograph of a leaf cross-section from a plant which had been in Fe^{59} labelled $FeCl_3$ for 180 minutes. Above, phase-contrast view of the tissue; below, bright-field view of the radioautograph. The cross-section shows a small vascular bundle consisting of two tracheary elements and some sieve elements. Most of the Fe^{59} was distributed in the sieve elements and border parenchyma, not in the tracheary elements. 480 : 1.

Fig. 5. Microradioautograph of a leaf cross-section from a plant which had been in Fe^{59} labelled $FeCl_3$ for 1,440 minutes. Above, phase-contrast view of the tissue; below, dark-field view of the radioautograph. The section shows an extended area of the leaf section, including a well developed vascular bundle; the greatest amounts of Fe^{59} were in the phloem and palisade parenchyma. 120 : 1.

Fig. 6. Microradioautographs of roots which had been in Fe^{59} labelled $FeCl_3$ for 14 hours. 6a is a radioautograph of a chemically fixed root, and gives an incorrect impression of the in vivo Fe^{59} distribution. 6b is a dry-radioautograph of a freeze-dried root. The dark nucleoli which result from improved staining (Delafield's hematoxylin) of the tissue (left) in 6b should not be confused with silver grains in the apposed radioautograph (right). 900 : 1.

3a

3b

3c

4

5

6a

6b
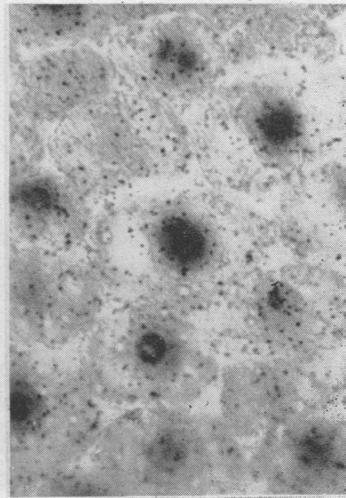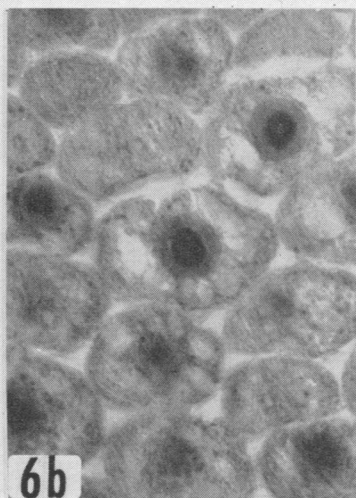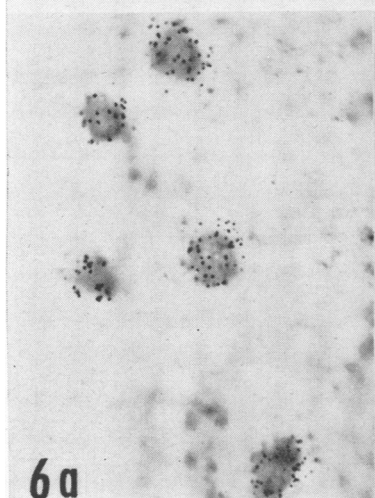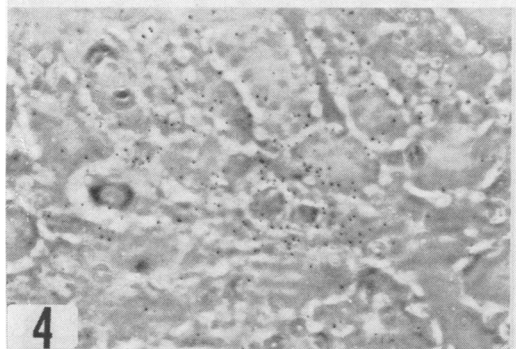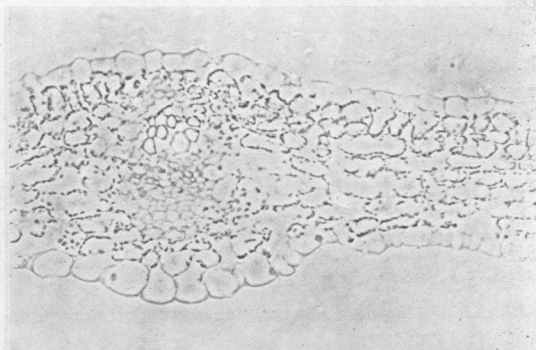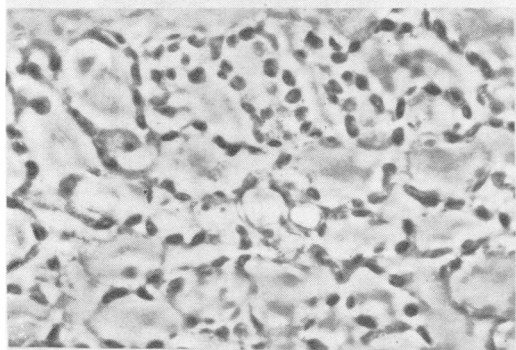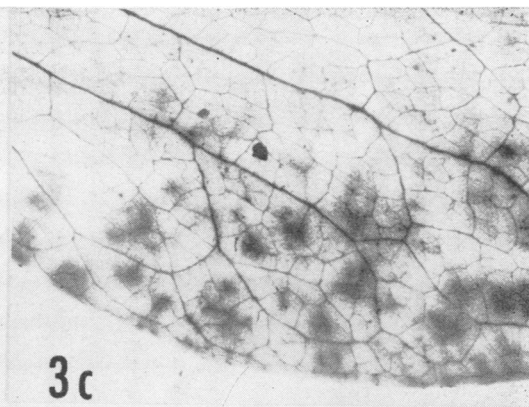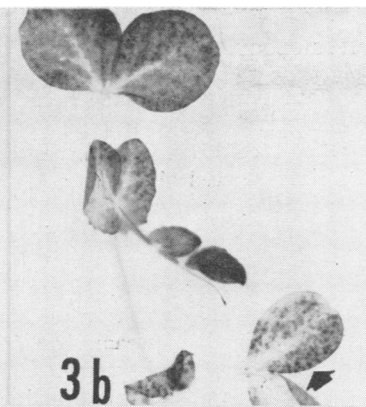
graphed. An enlarged image of one of the cleared leaves was projected on to an enlarged image of its radioautograph (fig 3c). Examination of this enlarged, superimposed leaf-image and radioautograph showed that the so-called spots observed in the radioautograph were regions of high Fe^{55} concentration associated with small veins and possibly vein endings.

Microradioautographs of similarly treated plants (placed in Fe^{59} rather than Fe^{55}) show that little of the labelled iron was in the xylem. The heavy deposits of labelled iron predicted by the macroradioautographs were found in border parenchyma surrounding the smaller veins and vein endings (fig 4) and in the phloem (fig 5). Plants which had been in Fe^{59} (0.2 $\mu c/\mu g$ Fe) for 1,440 minutes had a greater distribution of Fe^{59} outside the vascular bundles than plants which had been in Fe^{59} (0.5 $\mu c/\mu g$ Fe) for only 180 minutes. In the former, large amounts of Fe^{59} were observed in the pallisade parenchyma (fig 5).

The rather energetic β particles emitted by Fe^{59}, 0.27 Mev, 0.46 Mev and 1.56 Mev (the γ rays also emitted by Fe^{59} probably account for less than 1 % of the developable silver grains) limited resolution and made intracellular localization difficult. However, examination of a large number of radioautographs gave the impression that the Fe^{59} was uniformly distributed throughout the cytoplasm. This was in marked contrast to Possingham and Brown's (17) observation that Fe^{59} concentrated in the nuclei. To resolve this discrepancy, another experiment was performed in which their experimental conditions were duplicated. As suggested by Possingham and Brown, 2-day-old plants were placed in Fe^{59} (0.5 $\mu c/\mu g$ Fe) solutions in the dark at 25 C for 14 hours. Half the roots were prepared as described by Possingham and Brown. That is, they were chemically fixed in neutral formalin and radioautographed by the stripping-film method (16). The rest of the roots were frozen, freeze-dried, and dry radioautographed as in the other experiments above.

The Fe^{59} distribution in chemically fixed material (fig 6a) was identical to that observed by Possingham and Brown (17). The Fe^{59} was heavily concentrated in the nuclei. In freeze-dried material, the iron was uniformly distributed throughout the cytoplasm (fig 6b). It is probable that extensive leaching, or redistribution and leaching, of Fe^{59}, occurred in the chemically fixed materials. Therefore, conclusions based on the localization of iron by Possingham and Brown's procedures, or any other procedures using chemical fixatives in aqueous media, are questionable.

Discussion

A previous paper (5) presented evidence that iron transfer across the root to the stele is an active, metabolic process. It was also concluded that sufficient amounts of iron must accumulate in the root parenchyma before release to the xylem occurs. The radio-

autographs presented here substantiate these conclusions. Greater amounts of Fe^{59} were found in the root cells (but not necessarily xylem elements) of iron-starved plants (fig 1e) than in root cells grown in the presence of iron (fig 1b). The latter presumably had sufficient iron to meet their requirements and a large part of the Fe^{59} was transferred to the growing shoots (5). The iron-starved cells apparently did not transfer the incoming iron to the transpiration stream, but utilized the iron to meet their own requirements.

The radioautographs also confirm the hypothesis that roots placed in 0.5 ppm Fe as $FeCl_3$ are rapidly covered with iron particles (5). Figure 1d shows that roots grown in the absence of iron were completely coated with Fe^{59} after only 4 minutes in Fe^{59} Cl_3. Plants which had grown in the presence of $FeCl_3$ (and whose roots presumably had a coating of non-radioactive iron particles) had little Fe^{59} on their surface after 4 minutes in $Fe^{59}Cl_3$ (fig 1a). It is probable that the surface iron was not readily exchangeable with the iron in the nutrient solution. After 180 minutes, roots which had grown in the presence of iron still had less Fe^{59} on their surface (fig 1b) than roots which had grown in the absence of iron (fig 1e). Thus, exchange of iron previously on the root surface was incomplete even after 180 minutes in the experimental solution containing Fe^{59}.

In the leaves, most of the entering labelled iron was associated with the vascular bundles, especially the phloem and border parenchyma of small veins and vein endings. Subsequently, more of the labelled iron was found in the leaf mesophyll (figs 3, 4, & 5). This could indicate: A, iron moved up in the xylem, was rapidly absorbed by the phloem, and then slowly distributed from the phloem to other leaf cells, or B, iron moved up in the xylem, was rapidly absorbed by the phloem and slowly distributed from the xylem to the other leaf cells, or C, iron moved up in the phloem and was slowly distributed throughout the leaf from the phloem.

The experiments reported here do not distinguish among these possibilities. However, the last alternative is not easily reconciled with the marked effect transpiration has on iron transport (5). Nor is it in accordance with the presence of iron in exudates (presumably from the xylem) of decapitated plants (21, 22). But the possibility that iron moves from the roots to the shoots in the phloem requires further examination. Upward N transport in the phloem is believed to occur in horticultural species in which nitrate reduction occurs in the roots (8). If iron moves as a part of an organic molecule (21), it may also be translocated from the roots to the leaves in the phloem.

None of the plants we used were chlorotic, nor did they show any signs of becoming chlorotic. Though labelled iron accumulated rapidly in the vascular bundles and moved slowly to the surrounding mesophyll, this appears to be a characteristic of normal, healthy plants. When the iron supply is

limited, the phloem and border parenchyma—by virtue of their position or biochemical organization—may be the last to suffer iron deficiency.

Failure of iron to move from the vascular bundles does not necessarily indicate that it is precipitated or inactivated. Microradioautographs of normal leaves showed that little of the iron was in the xylem where other workers (14, 18) have assumed it was immobilized as precipitated insoluble phosphates. Though such precipitation may occur under conditions of high phosphate, concentrations of iron in the veins of chlorotic plants, such as found by Rediske and Biddulph (18), Rogers and Shive (20), and Olsen (14), probably reflect the ability of the phloem and border parenchyma to accumulate iron more rapidly than the mesophyll. Factors such as high phosphate may accentuate iron accumulation in the living cells of the vascular bundle rather than precipitate it in the tracheary elements.

Rapid iron absorption in one part of the plant may decrease iron movement to another part of the plant. In our experiments, for example, rapid uptake of labelled iron by starved root cells (fig 1) explained decreased Fe^{59} movement to the shoots (5). Under conditions of genuine iron deficiency this could mean that little or no iron reached the leaf mesophyll in spite of relatively large iron deposits in other tissues. It is frequently assumed that unequal distribution of iron in chlorotic plants demonstrates iron immobilization (14, 18, 20). However, such observations can also be interpreted as revealing the normal pattern of iron distribution. Accumulation of iron to meet the requirements of one group of cells to the detriment of another may be a consequence of the natural sequence of absorption and translocation. "Competition" roughly describes this phenomenon; the terms "inactivation" or "immobilization" should not be used in this context.

Summary

Macro- and microradioautographic procedures were employed to localize soluble as well as insoluble iron during the initial stages of its distribution in non-chlorotic plants.

Roots placed in 0.5 ppm Fe as $FeCl_3$ were rapidly covered with iron particles. Parenchyma cells in roots of iron-starved plants absorbed more iron and transferred less of it to the transpiration stream than did cells in roots which had previously received ample iron. 2,4-dinitrophenol inhibited the iron absorption.

In the leaves, much of the incoming iron was at first associated with the phloem and border parenchyma of vascular bundles. Though incoming iron accumulated in the vascular tissues, there was no evidence it was inactivated or precipitated in the xylem.

Rapid iron-absorption in certain plant tissues such as roots or vascular bundles was interpreted as a normal occurrence rather than as a pathological manifestation.

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